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Characterization of chrysanthemum (*Chrysanthemum grandiflorum*) varieties using ISSR markers

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ABSTRACT

Identification and characterization of new varieties is essential to meet DUS testing, address IPR issues and their utilization and conservation. A total of 68 ISSR primers were screened and out of these 23 primers which gave sufficient amplification were selected for the study. These primers produced 169 bands, out of which, 149 were polymorphic with a polymorphic percentage of 88.17%. The ISSR primers namely ISSR 22, ISSR 26, ISSR 38 generated least number of bands, i.e. four of which only two were polymorphic with a polymorphism of 50% in ISSR 22. The polymorphism across all the ISSR markers ranged between 66.7% (ISSR37) to 100% (ISSR 21, ISSR 25, ISSR 26, ISSR 28, ISSR 30 and ISSR 811). The resolving power of band (Rp) produced by ISSR markers ranged from 0.66 to 7.64 for primer ISSR 38 and ISSR 31 with a mean value of 86.48 for all the primers. The correlations between Rp and number of varieties identified by each ISSR primer (0.92) were fairly high. The value of polymorphic information content (PIC) was ranged from 0.023 (ISSR 809) to 0.553 (ISSR 26) with a mean value of 0.35 over all the primers. The UPGMA dendrogram based on ISSR analysis indicated that the outlier species were 74% dissimilar with other varieties and grouped separately. The cultivars Maghi Orange and Maghi Yellow had around 90% similarity and were close to cultivar Maghi White at over 80% similarity level. ISSR markers were proved to be useful for the characterization of the genotypes for their efficient utilization, management and IPR protection.

Key words: Chrysanthemum, Cluster analysis, Genetic diversity, ISSR markers, Polymorphism, PIC, Resolving power

Chrysanthemum is one of the most important ornamental crops grown worldwide as cut flower and pot mums. It holds a prominent position in both the domestic and international trade and ranks second after rose at the Dutch auctions (Steen 2010) indicating its global significance. The popularity of chrysanthemum is due to existence of diverse flower's size, shape and colour, vase life, plant architecture, hardiness and ease to grow under varied climatic conditions year round in one or the other part of the country. In India, it is reported to be cultivated in an area of about 18 360 ha with a production of 30.58 laks cut flower and 175 610 MT loose flower (NHB database 2013). It is reported that chrysanthemum is being bred in China and Japan for nearly 3000 years. In modern times, no single cultivar can sustain in market for a longer period, hence arises the need of development of novel varieties with quality traits and therefore a large number of varieties are being bred every year with desired characteristics and aesthetic values. Therefore, accurate identification and characterization of these varieties is essential to meet DUS testing, address IPR issues and to facilitate their utilization and conservation in crop improvement programme. One of the major drawbacks with morphological markers is their environment dependent expression, hence, they might not be appropriate for accurate analysis. Although isozyme markers are useful to characterize genetic diversity (Fiebich and Henning 1992, Roxas *et al.* 1993), and to identify the hybrids (Roxas *et al.* 1993), the paucity of isozyme loci restricts their usefulness in breeding (Helentjaris *et al.* 1986). Molecular markers give more reliable information for germplasm characterization and diversity analysis. Among the molecular markers, ISSR is the most widely exploited marker due to the fact that results are obtained quickly and are fairly inexpensive. The other major advantage of this approach lies in exploration of large genomic portions without prior sequence information and requires small quantity of DNA. The ISSR technique is a PCR based method, which involves amplification of DNA

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segment present at amplifiable distance in between two identical micro satellite repeat regions oriented in opposite direction. The technique uses microsatellite, usually 16-25 bp long as primer in single PCR reaction targeting multiple genomic loci to amplify mainly inter simple sequence repeat region (ISSR) sequences of different sizes. Though vast diversity exists in chrysanthemum, there are only a few reports on the use of molecular markers for diversity analysis in chrysanthemum. So keeping this in view, the present investigations were undertaken to characterize the chrysanthemum varieties using ISSR markers.

MATERIALS AND METHODS

The experimental material comprised 75 genotypes of chrysanthemum (Table 1). The investigations were carried out at the Division of Floriculture and Landscaping, Indian Agricultural Research Institute, New Delhi and diversity analysis using molecular markers was done at National Bureau of Plant Genetic Resources, New Delhi during 2009.

Table 1 List of *Chrysanthemum* varieties utilized for molecular characterization

Varieties	Varieties
Kanchil	Pusa Anmol
Gulmohr	Yellow Bangla
Shyamal	Sharad Mala
Sadwin Yellow	Star White
White Andaman	TERI
Aparjita	Poornima White
Sadbhawna	Waters May
Flirt	Beauty
Neelima	Jubilee
White Prolific	Maghi Orange
Ravikiran	Maghi White
Birbal Sahni	Maghi Yellow
Shukla	Kalvin Orange
Yellow Charm	Diana
Pink Cloud	Pankaj
Kajole	Kalvin Pink
Gaity	Sonali Tara
Geetanjali	Mother Teressa
Star Pink	Pinked White
Korean Small	Gajra
Yellow Star	Santa Dine
Ajay	Red Shringar
Meghavi	Shwet Shringar
Yellow Gold	White Anemone
Lalpari	Mahatma Gandhi
Red Gold	Raja Orange
Vasantika	Tokyo Soldier
Kundan	Korean Small
Kargil	Kalvin Yellow
Shanti	Greenish White
Taichen Queen	Ajay
Star Yellow	Texas Gold
Snowball	Yellow Reflex
Presiden Viger	Golden Yellow
Jayanti	Red D spoon
Dolly Orange	Annual Chrysanthemum
Liliput	Annual Chrysanthemum

Total genomic DNA was extracted from five g of young leaf tissues using CTAB method as given by Saghai-Maroof *et al.* (1984). The DNA was purified and further quantified by using DyNA Quant Fluorimeter. Each part of DNA sample was diluted with 10:1 TE solution to yield a working concentration of 5 ng/ml.

To determine optimal amplification reaction conditions, a factorial experiment was carried out at three concentrations of each of $MgCl_2$ (2.0 mM, 2.5 mM and 3.0 mM), *Taq* DNA polymerase (0.5 U, 0.75 U and 1.0 U), template DNA (10 ng, 20 ng and 30 ng), and 0.5 mM of primer in a volume of 25ml at different annealing temperatures. PCR reaction was carried out in a Peltier Thermal Cycler PTC-200 (M/s M J Research). The conditions for amplification of bands were optimized where denaturation was done at 95°C for 5 minutes; followed by 40 cycles at 94°C for 1 minute and AT°C (varied annealing temperature with each primer); 72°C for 2 minutes and a final extension at 72°C for 7 minutes. A random set of 68 primers were used for the ISSR analysis (Zietkiewick *et al.* 1994). Out of these, 23 primers which gave sufficient polymorphism were used for assessing variation in 75 genotypes. The details of these twenty-three primers are listed in Table 2. The amplified products were resolved by electrophoresis in 2% agarose gel run in 1X

Table 2 List of ISSR primers selected for PCR amplification and corresponding annealing temperature

Primer	Tm*	Sequence (5′-3′)
ISSR 6	63.4°C	$C(CCT)_4TC$
ISSR 8	53.4°C	$(GTGA)_4$
ISSR 10	53.4°C	$(GA)_9AT$
ISSR 15	56.7°C	$(GA)_9C$
ISSR 16	59.6°C	$(GA)_9T$
ISSR 21	59.6°C	$(AC)_9C$
ISSR 22	47°C	$(AC)_9T$
ISSR 24	47°C	$(TC)_9A$
ISSR 25	63.4°C	$(TC)_9C$
ISSR 26	47°C	$(TC)_9T$
ISSR 27	47°C	$(TC)_9G$
ISSR 28	53.4°C	$(AG)_8AC$
ISSR 30	65°C	$(CA)_8TC$
ISSR 31	50.5°C	$(CT)_8GA$
ISSR 33	50.5°C	$(TG)_8AC$
ISSR 34	56.7°C	$(CA)_8TG$
ISSR 35	45.5°C	$(CT)_8AG$
ISSR 36	47°C	$(AG)_8CT$
ISSR 37	50.5°C	$(GA)_8TC$
ISSR 38	47°C	$(AC)_8$ CG
ISSR 809	50°C	$(AG)_8G$
ISSR 810	50°C	$(GA)_8T$
ISSR 811	50°C	$(GA)_8C$

*T_m, Melting temperature

Table 3 'Generated Fragments' statistics and number of varieties identified by ISSR primers

ISSR Primer	No. of Bands	No. of polymorphic bands	% polymorphism	Rp	PIC=2*pi*qi	Gene Div.	No. of varieties identified
ISSR 8	8	7	87.5	4.41	0.252	0.947	10
ISSR 10	9	8	88.89	4.89	0.342	0.947	19
ISSR 15	7	6	85.71	2.34	0.437	0.909	5
ISSR 16	6	5	83.33	2.64	0.372	0.799	2
ISSR 21	7	7	100	3.64	0.388	0.932	7
ISSR 22	4	2	50	1.78	0.424	0.935	0
ISSR 24	5	4	80	1.95	0.418	0.932	3
ISSR 25	6	6	100	3.29	0.232	0.894	10
ISSR 26	4	4	100	1.21	0.553	0.906	1
ISSR 27	5	4	80	2.59	0.365	0.944	1
ISSR 28	9	9	100	2.79	0.457	0.9	1
ISSR 30	11	11	100	5.09	0.292	0.967	32
ISSR 31	14	13	92.86	7.64	0.289	0.954	47
ISSR 33	6	5	83.33	3.08	0.322	0.941	6
ISSR 34	8	6	75	3.32	0.386	0.913	6
ISSR 35	11	10	90.91	6.34	0.296	1.048	36
ISSR 36	7	6	85.71	4.04	0.285	0.953	10
ISSR 37	6	4	66.67	2.82	0.288	0.94	3
ISSR 38	4	3	75	0.66	0.395	0.715	1
ISSR 809	11	10	90.91	5.75	0.023	0.086	38
ISSR 810	8	7	87.5	2.61	0.288	0.653	4
ISSR 811	6	6	100	2	0.488	0.912	3
ISSR 812	7	6	85.71	3.77	0.357	0.942	8
Total	169	149	88.17				
Min	4	2	66.67	0.66	0.023	0.086	0
Max	14	13	100	7.64	0.553	1.048	47
Mean	7.35	6.48	86.48	3.42	0.35	0.87	11

TBE buffer and amplified fragments were visualized by ethidium bromide staining. The resolved product was photographed under UV light using gel documentation FlourchemTM 5500 (Alpha InfoTech USA.).

The scoring of reproducible DNA bands (bands present in both repetitions of an individual sample) was done manually. Weak bands with negligible intensity were excluded from the final data analysis. The scoring of band profiles for each parent was carried out in a binary mode (1 indicating its presence: 0 indicating its absence). Similarity index values for ISSR patterns were calculated for all the possible pair wise comparisons, using Jaccard's (genetic) similarity coefficient (GS): a/(n-d), Where 'a' is the number of positive matches; 'd' is the number of negative matches and 'n' is the total sample size (Jaccard 1908). The resolving power for each primer was calculated using formula, Rp = $\sum I_b$, where I_b = 1-{2x (0.5-p)}, where 'p' is the proportion of the 15 genotypes containing the bands (Prevost and Wilkinson 1999).

The basic information that determines their application in genetic mapping, the marker systems ISSR, were calculated by using the Polymorphism information content (PIC) (Lynch and Walsh 1998). It was calculated by using formula, PIC = 1-$\sum pi^2$, where pi is the frequency of the i^{th} alleles. Computation for multivariate analysis was done using NTSYS-pc Version 2.02 (Numerical Taxonomic System) software (Rohlf 2000) and similarity matrix was subjected to the cluster analysis of Unweighted Paired Group method using Arithmetic Averages (UPGMA) and dengrogram was constructed.

RESULTS AND DISCUSSION

ISSR polymorphism

Sixty eight primers were tested in ISSR reactions and primers producing banding patterns that were difficult to score and failed to amplify consistently in all the genotypes were excluded. Thus, 23 of these primers were chosen for the estimation of genetic similarities among the 75 varieties of chrysanthemum. The UPGMA Dendrogram on genetic similarity based on ISSR markers are presented in Fig 1. Representative banding patterns, as observed with primer ISSR 21 and ISSR 31 are shown in Fig 2 and 3, respectively. The lane number given in Fig 2 and 3 corresponds to the serial number of the varieties listed in Table 1. A total of 23 ISSR primers used generated 169 bands out of which 149 were polymorphic with a polymorphic percentage of 88.17%. The ISSR primer ISSR 22, ISSR 26, ISSR 38 generated least number of bands, i.e. four of which only two were polymorphic with a polymorphism of 50% in ISSR 22. The

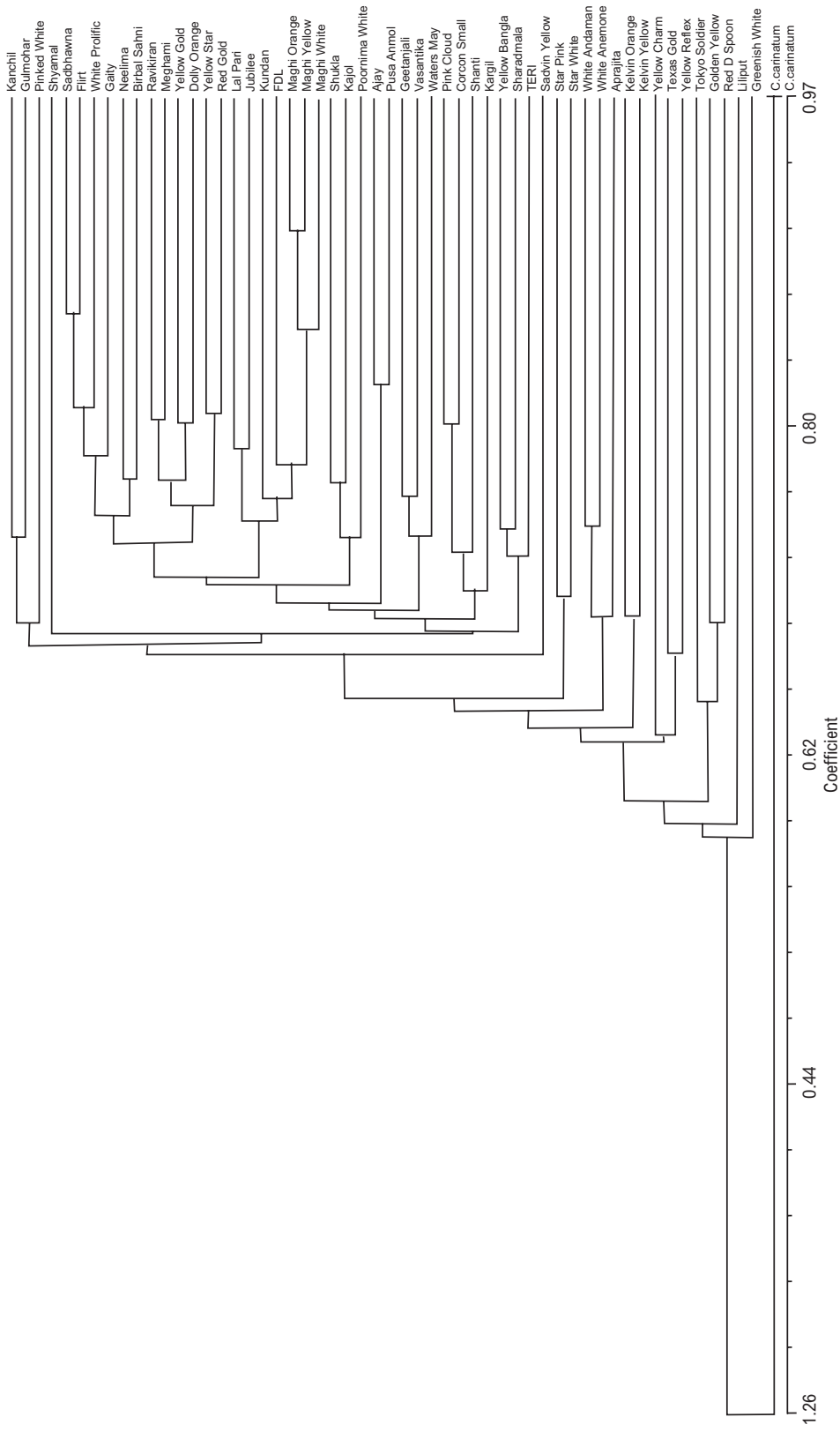

Fig 1 UPGMA dendrogram on genetic similarity based on ISSR markers

primer ISSR 31 generated maximum number of bands, i.e. 14 of which 13 were polymorphic with 92.85% polymorphism. The polymorphism across all the ISSR markers ranged between 66-67% (ISSR37) to 100% (ISSR 21, ISSR 25, ISSR 26, ISSR 28, ISSR 30 and ISSR 811). The mean values of all the ISSR primers were 7.35 for number of bands generated, 6.48 for number of polymorphic bands and 86.48 for per cent polymorphism.

The resolving power of band produced by ISSR markers ranged from 0.66 to 7.64 for primer ISSR 38 and ISSR 31 with a mean value of 86.48 for all the primers. The use of primers having high Rp values may be used for varietal identification rather using arbitrarily more primers thus save time and resources. The correlations between Rp and number of varieties identified by each ISSR primer (0.92) were fairly high. The value of polymorphic information content (PIC) was ranged from 0.023 (ISSR 809) to 0.553 (ISSR 26) with a mean value of 0.35 over all the primers. The estimates for number of varieties identified by ISSR primers ranged between 0 (ISSR 22) to 47 (ISSR 31). The other promising ISSR primers identifying fairly high number of varieties were ISSR 809 (38), ISSR 35 (36) and ISSR 10 (32). ISSR primers were also found to be very informative for studying polymorphism in marigold genotypes (Namita *et al.* 2013, Zeng *et al.* 2010, Panwar *et al*. 2013, Qi *et al*. 2007), in chrysanthemum (Baliyan *et al.* 2014, Mukherjee *et al.* 2013, Palai and Rout 2011) and in wild sunflower (Garayalde *et al.* 2011).

ISSR based genetic diversity and clustering pattern of chrysanthemum varieties

Based on Jacards similarity coefficients, genetic similarities ranged from 0.26-0.97 among all varieties. The

UPGMA dendogram based on ISSR analysis indicated that the outlier species was 74% dissimilar with other varieties and grouped separately. The cultivar Maghi Orange and Maghi Yellow had around 90% similarity and were close to cultivar Maghi White at over 80% similarity level. The other varieties sharing higher similarity levels were flirt with Yellow Gold, Pink Cloud with Corcon small and Sadbhawna with Jubilee. The varieties Red D Spoon, Yellow Reflex and Tokyo Soldier grouped together and showed maximum dissimilarity of approximately 45% with other varieties. Similar studies on clustering pattern in other varieties of chrysanthemum were also conducted by various researchers (Baliyan *et al.* 2014, Mukherjee *et al.* 2013, Palai and Rout 2011). The systematic characterization is the backbone of chrysanthemum germplasm management and its conservation, IPR protection, DUS testing, discriminate accessions, monitoring genetic integrity during long term conservation and for assessment of diversity. Awareness and need for IPR protection and large number of varieties submitted for registration have caused an increase in the size of reference collections of varieties, required to be grown and tested to ensure that candidate variety is distinct. The genotypes were found to be diverse based on ISSR markers and can be further utilized in crop improvement programmes. The high level of genetic variability among the genotypes would be useful for selecting parents in the development of new varieties.

The foregoing results and discussion indicated that ISSR profiling offered an effective means of assessing genetic variation and thus would be useful for differentiation of elite breeding lines and varieties. Conversion of specific ISSR segments into SCAR (Sequenced characterized amplified region) markers could enhance the value of these markers for varietal identification. The varieties were found to be diverse based on ISSR markers and could be utilized in conservation biology and crop improvement programmes. The high level of genetic variability among varieties would be useful for selecting parents in the development of new varieties.

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